

Gene expression variance based on random sequencing in rat remnant kidney

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Background. Several examinations have been performed to identify the genes involved in chronic renal failure using 5/6 nephrectomized rats. Recently, many systematic techniques for examining molecular expression have been developed. They might also be effective in elucidating the molecular mechanism of progressive renal failure. In this study, digital expression profiling was carried out to construct a subtractive mRNA expression database for the 5/6 nephrectomized kidney.

Methods. One thousand clones were randomly sequenced from 5/6 nephrectomized and sham-operated rat kidney cDNA libraries, respectively, and defined by BLAST search. In silico subtractive analysis was performed to search for genes up- or down-regulated in the 5/6 nephrectomized kidney.

Results. The growth factor-related mRNAs and the mRNAs encoding cytoskeletal or membrane proteins were up-regulated, but the transporter-related mRNAs were down-regulated in the 5/6 nephrectomized kidney database. In silico subtraction revealed that 63 mRNAs were increased and 59 were decreased in the 5/6 nephrectomized kidney. To confirm whether the in silico subtractive database reflected the actual expression of mRNA or protein, 12 known genes were examined by Northern blotting or immunoblotting, respectively. The actual expression of the 12 genes was comparable with the results of in silico subtraction. In addition, we successfully isolated five unknown genes, two up-regulated and three down-regulated in the 5/6 nephrectomized kidney.

Conclusion. We constructed a subtractive mRNA expression database for 5/6 nephrectomized kidney, which reflects the actual alterations in mRNA expression after subtotal nephrectomy. This database may be useful for elucidation of the molecular mechanism of progressive renal failure.

Five-sixth nephrectomized rats are widely used as a model of progressive renal failure [1, 2]. There are several

findings in which specific genes have been up-regulated or down-regulated in the 5/6 nephrectomized kidney. One to 4 weeks after 5/6 nephrectomy, the genes related to hypertrophy, development of glomerular sclerosis, or vascular tone, such as transforming growth factor β (TGF- β), insulin-like growth factor (IGF) and so on, are up-regulated in the remnant kidney [3–8]. On the other hand, organic ion transporters OAT-K and OCT-2 in proximal tubules were down-regulated in the 5/6 nephrectomized kidneys [9, 10]. However, the kidney is a heterogeneous tissue with many types of cell and the progression of chronic renal failure (CRF) involves many factors. Therefore, analysis of overall genes should be done to understand further the molecular mechanisms of progressive CRF.

To date, many systematic techniques have been developed to analyze molecular expression. Polymerase chain reaction (PCR)-coupled representational difference analysis (RDA-PCR) [11], differential display [12], cDNA microarray [13, 14], and so on are examples. Digital expression profiling is one of the methods of large-scale gene expression analysis in which the mRNA population in a given tissue is assessed quantitatively by sequencing randomly selected clones from a 3'-directed cDNA library [15, 16]. Recently, Takenaka et al [17, 18] constructed cDNA databases for mouse proximal tubules and collecting ducts, and successfully obtained information on the expression profile of normal kidney.

The isolation of genes up-regulated in 5/6 nephrectomized mouse kidneys using RDA-PCR was previously reported by Zhang et al [19]. Ten known genes and nine novel genes were isolated in that study. Although this method is advantageous for screening apparently up-regulated genes, it is difficult to isolate all up-regulated genes unless thousands of clones are screened [19]. In addition, the relative expression level could not be determined by this method. Taken together, RDA-PCR would certainly be effective for searching for up-regulated genes; however, it would be insufficient for screening for massive physiologic variation in progressive

Key words: expression profiling, in silico subtraction, database, chronic renal failure, 5/6 nephrectomized rats.

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renal failure. Therefore, we adopted the digital expression profiling to identify up- or down-regulated genes simultaneously in progressive renal failure.

Recently we have identified an Na⁺-dependent glucose transporter (rNaGLT1) from a rat kidney cDNA library by the digital expression profiling [20]. rNaGLT1 is abundantly expressed and considered to be critical for glucose and fructose reabsorption in renal proximal tubules [20, 21]. Thus, this method would be useful for cloning novel genes, which are abundantly expressed and are physiologically important. Applying this method to disease models may be beneficial for identifying some genes related to pathophysiologic status. In this study, we constructed a cDNA database of 5/6 nephrectomized and sham-operated rat kidneys 2 weeks after 5/6 nephrectomy by this method. The aim of this study was to construct a subtractive mRNA expression database for the 5/6 nephrectomized kidney, and find novel genes up- or down-regulated by subtotal nephrectomy.

METHODS

Materials

Vancomycin and cisplatin were purchased from Shionogi Co. (Osaka, Japan) and Bristol-Myers Squibb Co. (Tokyo, Japan), respectively. Protease inhibitor cocktail was obtained from Calbiochem (San Diego, CA, USA). Antirat osteopontin antibody was from Immuno Biological Laboratories Co. (Gunma, Japan). Antirat cathepsin B antibody and antimouse Na⁺-K⁺ATPase α 1 subunit antibody were from Upstate Biotechnology Co. (Lake Placid, NY, USA). All other chemicals were of the highest purity available.

Animals

Male Wistar albino rats were purchased from SLC Animal Research Laboratories, and cared for in accordance with the *Guidelines for Animal Experiments of Kyoto University*. The animals were fed normal pellet food ad libitum, and given water freely. To induce 5/6 nephrectomy, 7-week-old male rats weighing 200 to 220 g were surgically operated as previously described [9, 10]. Briefly, the right kidneys were removed, and the posterior and anterior apical segmental branches of the left renal artery were individually ligated. Two weeks after surgery, the rats were placed in metabolic cages for the collection of 24-hour urine samples used for measurements of urinary creatinine and *N*-acetyl- β -D-glucosaminidase (NAG) excretion. Blood was collected from a cervical vein for the measurement of plasma creatinine and blood urea nitrogen (BUN). Creatinine was measured using the Jaffé method with commercial kits (Wako Pure Chemical Industries, Osaka, Japan). BUN was measured with i-STAT[®] Portable Clinical Analyzer (i-STAT Co., East Windsor, NJ, USA) as described elsewhere [22]. NAG

was measured using commercial kits (Shionogi Co.). Urinary albumin was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Nephra II) (Exocell, Inc., Philadelphia, PA, USA).

To induce nephrotoxicity with cisplatin or vancomycin, 9-week-old male rats weighing 290 to 310 g were administered cisplatin intraperitoneally (6 mg/kg) or vancomycin intravenously (500 mg/kg) [23]. Vehicle control rats were administered saline intravenously (10 mL/kg). Two days after the administration, the rats were placed in metabolic cages, and biochemical parameters of blood and urine were measured as described above.

Construction of a rat kidney cDNA library

Rat kidney total RNA was extracted from eight 5/6 nephrectomized or four sham-operated rats by guanidine isothiocyanate-CsCl ultracentrifugation, 2 weeks after nephrectomy. The poly(A)⁺ RNA was purified with oligo(dT)-cellulose (Stratagene, La Jolla, CA, USA) affinity column chromatography, as described previously [24]. The creatinine clearance of 5/6 nephrectomized rats used in the construction of the cDNA library was significantly lower than that of sham-operated rats (5/6 nephrectomized rats 2.0 ± 0.2 mL/min/kg, sham-operated rats 4.1 ± 0.5 mL/min/kg, mean \pm SE of eight and four rats, respectively). After confirmation of induction of the appropriate nephropathy by creatinine clearance, the poly(A)⁺ RNA isolated from sham-operated or 5/6 nephrectomized rat kidneys was used to construct the cDNA libraries. The libraries were constructed using the λ ZAP Express cDNA Synthesis Kit (Stratagene) according to the manufacturer's instructions. Briefly, cDNA was synthesized using a linker primer, and digested by the restriction enzyme *Xho*I. To remove excess adaptor and to concentrate the complete length of cDNA, the cDNA was size-fractionated with cDNA Size Fraction Columns (Invitrogen Life Technology Co., Carlsbad, CA, USA). The cDNA was ligated in λ ZAP expression vector and excised out of the phage in the form of the pBK-cytomegalovirus (CMV) phagemid vector. The phagemid vectors with the cDNA inserts were transferred into *Escherichia coli* (strain XL0LR). From these cDNA libraries, 1033 (5/6 nephrectomized kidney) and 1015 (sham-operated kidney) randomly selected colonies were sequenced using a Multi-Capillary DNA Sequencing System RISA-384 (Shimadzu Co., Kyoto, Japan).

Data analysis

The isolated clones were partially sequenced (500–550 bp) from the 5' end using T3 primer, and the resulting sequences were searched against GenBank, EMBL, DDBJ, and PDB using the BLAST program. If the Expect value (E value) was less than 10^{-50} ($1e-50$), the clone was regarded as representing the corresponding gene. A clone with an E value of more than $1e-50$ was identified as

a “novel gene.” The novel genes were further sequenced from the 3' end using T7 primer. The poly(A)⁺ tail was eliminated from the sequencing data. If overlapping regions had more than 90% homology, the two sequences were considered identical. The subtractive database of the 5/6 nephrectomized kidney and the sham-operated kidney was created *in silico*. The criteria of a significant difference in the subtractive difference was defined as follows; a frequency in one database was threefold higher than that in the other database and the frequency in the subtractive database was required at least two.

Western blotting

The rat whole kidney was homogenized in sucrose buffer (250 mmol/L sucrose, 5 mmol/L Tris-Hepes, pH 7.4, and 1% protease inhibitor cocktail). The tissue lysate was sonicated and clarified by centrifugation [lysis buffer 50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L ethyleneglycol tetraacetate (EGTA), and 1% protease inhibitor cocktail]. The crude membrane fractions of the rat kidney were prepared as described [25]. The kidney lysate was used for analysis of cathepsin B, and the membrane fraction was used for osteopontin, OAT1, and the Na⁺-K⁺ ATPase α 1 subunit. The lysate (20 μ g) or the membrane fraction (25 μ g) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond[®]-PVDF) (Amersham Biosciences, Piscataway, NJ, USA) by semidry electroblotting for 35 minutes. The blots were blocked, washed, and incubated with the primary antibodies overnight at 4°C. The bound antibody was detected on x-ray film by enhanced chemiluminescence (ECL) with a horseradish peroxidase-conjugated antirabbit or antimouse IgG antibody and cyclic diacylhydrazides (Amersham Biosciences).

Northern blotting

The rat kidney total RNA was extracted using RNeasy[®] kit (Qiagen, Inc., Hilden, Germany), according to the manufacturer's directions. Five micrograms of total RNA was electrophoresed in a 1% denaturing agarose gel containing formaldehyde and transferred onto Hybond[®] N⁺ nylon membranes (Amersham Biosciences). The transferred RNAs were linked to the nylon membrane by an ultraviolet cross linker. The quality of RNA was assessed by ethidium bromide staining. After transfer, the blots were hybridized under high stringency conditions [50% formamide, 5 \times sodium chloride sodium phosphate EDTA buffer (SSPE)] [20 \times SSPE; 3 mol/L NaCl, 0.2 mol/L NaH₂PO₄, and 0.02 mol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4], 5 \times Denhardt's solution, 0.1% SDS, and 10 μ g/mL of herring sperm DNA at 42°C with each cDNA insert labeled with [α -³²P]

deoxycytidine triphosphate (dCTP) (29.6 TBq/mmol) (Amersham Biosciences). After hybridization, the blots were washed three times with 2 \times sodium chloride sodium citrate buffer (SSC) (20 \times SSC; 3 mol/L NaCl and 0.3 mol/L sodium citrate, pH 7.0) containing 0.1% SDS at 42°C for 10 minutes, and then twice with 0.5 \times SSC/0.1% SDS at 42°C for 30 minutes. The dried membranes were exposed to the imaging plates of Fujix BIO-imaging Analyzer BAS-2000 II (Fuji Photo Film Co., Tokyo, Japan).

As nonspecific hybridization was observed in the detection of 4F2hc mRNA using full-length probe, Northern blotting of 4F2hc was performed with a digoxigenin (DIG)-labeled probe corresponding to nucleotide positions 660–1074. The probe was labeled with DIG using PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Hybridization was performed as described above. After hybridization, the blots were washed twice with 2 \times SSC/0.1% SDS for 10 minutes, and then three times with 0.5 \times SSC/0.1% SDS for 20 minutes. DIG-labeled nucleic acids were detected using a DIG luminescent detection kit (Roche Diagnostics), according to the manufacturer's directions.

Microdissection of rat nephron segments

The rat nephron was microdissected as described previously [26]. Briefly, the left kidneys of 5/6 nephrectomized and sham-operated rats 2 weeks after subtotal nephrectomy were perfused and removed. Slices were cut along the medullary axis and incubated with collagenase. The tubules were microdissected to obtain the following structures: glomerulus, proximal convoluted tubule (PCT), proximal straight tubule (PST), medullary thick ascending limb (MAL), cortical thick ascending limb (CAL), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). After microdissection, 20 glomeruli and 8 mm of each dissected tubule segment were transferred into tubes to isolate total RNA using RNeasy[®] minikit (Qiagen).

RT-PCR analysis

Total RNA from the dissected tubules and poly(A)⁺ RNA from rat tissues (brain, heart, lung, liver, small intestine, spleen, kidney cortex, and kidney medulla) was reverse-transcribed with random hexamers, using Superscript II reverse transcriptase (Invitrogen), followed by RNase H (Invitrogen) digestion. These single-stranded DNA fragments were amplified with primer sets specific for the five unknown genes.

Analysis of novel or unknown genes

There were 343 mRNAs in which the nucleic acid and amino acid sequences have been clarified with little

information available concerning their functions and tissue distributions (about 17% of 2048 clones sequenced). We identified them as “unknown genes.” There were 18 novel or unknown genes appearing twice either in the 5/6 nephrectomized or in the sham-operated kidney. Among them, five genes were fully sequenced because significant differences in expression were shown between the 5/6 nephrectomized and sham-operated kidney by Northern blotting. To analyze the 5' upstream region, a 5' rapid amplification of cDNA ends (RACE) was performed using Marathon-Ready cDNA kits (Clontech Laboratories, Inc., Palo Alto, CA, USA). The motifs of their deduced amino acid sequences were searched against Smart (Simple Modular Architecture Research Tool, *smart.embl-heidelberg.de/*) and Pfam (Protein families database of alignments and HMMs, *pfam.wustl.edu/*) libraries. In addition, the tissue distribution, the localization along the nephron, the expression in the earlier phases of 5/6 nephrectomized rats, and the expression in acute nephropathy induced by cisplatin or vancomycin of these five unknown genes were examined.

Statistical analysis

Data are expressed as the mean \pm SEM. Data were analyzed statistically by the unpaired Student *t* test, or by a one-way analysis of variance (ANOVA) followed by Fisher's *t* test, when multiple comparisons against the control were needed. Significance was set at $P < 0.05$.

RESULTS

Gene expression profiles and in silico subtraction

In the 5/6 nephrectomized kidney, there were 41 genes highly expressed (more than three times), 84 genes expressed twice, and 663 genes expressed once. In total, 788 genes were found. In the sham kidney, there were 46 genes highly expressed, 79 genes expressed twice, and 594 genes expressed once, a total of 729. These isolated genes were classified according to function and localization. The results are shown in Table 1. In the 5/6 nephrectomized kidney, the growth factor-related mRNA and the mRNA encoding cytoskeletal or membrane proteins were increased compared with those in the sham-operated kidney. In contrast, the genes related to the transporter were decreased in the 5/6 nephrectomized kidney.

In the 5/6 nephrectomized kidney, 853 clones were identical to previously reported genes, but 168 clones among them were classified as unknown genes. There were 180 clones for which the E value of the BLAST homologue analysis was more than $1e-50$ and thought to be novel genes. Among them, 12 clones were considered to be duplicated, as their sequences at the 3' end were identical. As a result, we obtained 174 novel genes in the 5/6 nephrectomized kidney. In the sham-operated

Table 1. Classification of mRNA in the 5/6 nephrectomized and sham-operated kidney according to function or localization

Category	5/6 Nephrectomy frequency	%	Sham-operated frequency	%
Cytoskeletal, membrane protein	73	7.1	37	3.6
Growth factor-related	15	1.5	4	0.4
Ribosome	27	2.6	19	1.9
Heat shock protein	5	0.5	7	0.7
Metabolic enzyme	29	2.8	31	3.1
Enzyme	183	17.7	227	22.4
Protease	29	2.8	27	2.7
Nuclear factor	66	6.4	54	5.3
Mitochondria	53	5.1	46	4.5
Signal transduction	54	5.2	52	5.1
Transporter	24	2.3	37	3.6
Receptor channel	17	1.6	14	1.4
Endocytosis	15	1.5	15	1.5
Other	95	9.2	106	10.4
Unknown; function unknown	168	16.3	175	17.2
Unknown; novel	180	17.4	164	16.2
Total	1033	100.0	1015	100.0

Frequency and percentages of clones classified into 16 categories are listed. “Other” means a clone not classified into the above 13 categories.

kidney, 851 clones were identical to previously reported genes, but 175 clones among them were unknown genes. There were 164 clones classified as novel genes. Among them, 14 clones were judged to be duplicated, based on the sequence of the 3' end. As a result, 157 novel genes were observed in the sham-operated kidney.

In silico subtraction of the 5/6 nephrectomized and the sham-operated kidney databases was performed. Sixty-three genes were increased (Table 2) and 59 decreased in the 5/6 nephrectomized kidney (Table 3).

Confirmation of the database

To confirm whether the in silico subtractive database of 5/6 nephrectomized kidney reflected the actual expression of mRNA, the expression of 12 genes which was considered to be common in the kidney was examined by Northern blotting in five 5/6 nephrectomized and sham-operated rats at 2 weeks after surgery. A summary of the 12 genes is described in Table 4. Because the mRNA database to reflect not only mRNA expression level but also protein expression level is important to elucidate the mechanisms of progressive nephropathy, the expressional changes of cathepsin B, osteopontin, OAT1, and the Na^+/K^+ ATPase α_1 subunit, whose specific antibodies were available, were also examined by Western blotting. The blood and urine biochemical parameters and kidney weights are shown in Table 6. Decreases in creatinine clearance and albuminuria were found in the 5/6 nephrectomized rats, but urinary NAG excretion, an indicator of proximal tubule injury, was not significantly changed.

Table 2. The mRNAs up-regulated in the 5/6 nephrectomized (NR) kidney database by in silico subtraction

Frequency NR	Sham- operated	Gene name	Category	Accession number
5	0	Rat liver aldolase B mRNA, complete cds	Enzyme	M10149
5	0	Rattus norvegicus cytochrome b, mRNA	Mitochondria	AF295545
4	0	Rat mRNA for sulfated glycoprotein 2	Other	AB013455
4	0	Rattus norvegicus cathepsin B (Ctsb), mRNA	Protease	X13231
4	0	Rattus norvegicus mRNA for NaPi-2 gamma, complete cds	Transporter	NM_022597
4	1	Rattus norvegicus cytochrome P450, 4a10 (Cyp4a10), mRNA	Metabolic enzyme	NM_031605
4	1	Rattus norvegicus eukaryotic translation elongation factor 2 (Eef2), mRNA	Nuclear	NM_017245
3	0	Rattus norvegicus CD24 antigen (Cd24), mRNA	Other	NM_013457
3	0	Rattus norvegicus kidney injury molecule-1 (KIM-1), mRNA, complete cds	Other	BC006660
3	0	Rattus norvegicus 3-hydroxyanthranilate 3,4-dioxygenase (Haao), mRNA	Enzyme	NM_020076
3	0	Rattus norvegicus glutamate-cysteine ligase regulatory, mRNA	Enzyme	NM_012752
3	0	Mus musculus adducin 1 (alpha) (Add1), mRNA	Cytoskeletal, membrane protein	AF219904
3	0	Rattus norvegicus folate binding protein, mRNA, complete cds	Cytoskeletal, membrane protein	NM_017305
3	0	Rattus norvegicus Sialoprotein (osteopontin) (Spp1), mRNA	Cytoskeletal, membrane protein	AF035963
3	0	Rattus norvegicus ubiquitin C (Ubc), mRNA	Protease	NM_017341
3	0	Rattus norvegicus similar to NADH-ubiquinone oxidoreductase 75 kD subunit	Mitochondria	NM_012881
3	1	Rat leukemia virus gag (gag), polymerase, and envelope protein genes, complete cds	Enzyme	AK004990
3	1	Rat mRNA for preprocathepsin D (EC 3.4.23.5)	Protease	M77194
3	1	Rattus norvegicus similar to claudin 2	Cytoskeletal, membrane protein	XM236535
2	0	Mus musculus alpha-2 type IV collagen, mRNA, complete cds	Other	J04695
2	0	Mus musculus interferon regulatory factor 3 (Irf3), mRNA	Other	NM_016849
2	0	Mus musculus procollagen, type VI, alpha 1 (Col6a1), mRNA	Other	NM_009933
2	0	Rattus norvegicus peroxiredoxin 3 (Prdx3), mRNA	Other	NM_022540
2	0	Mus musculus, ELK3, member of ETS oncogene family, clone MGC:11528	Other	BC005686
2	0	HSD IV = peroxisome proliferator-inducible gene [rats, F344, liver, mRNA partial, 2480 nt]	Enzyme	S83279
2	0	Mus musculus serine hydroxymethyltransferase, mRNA, complete cds	Enzyme	AF237702
2	0	Rat mRNA for glutamine synthetase (EC 6.3.1.2.)	Enzyme	X07921
2	0	Rattus norvegicus cysteine desulfurase (NifS), mRNA, complete cds	Enzyme	AF336041
2	0	Rattus norvegicus D-dopachrome tautomerase (Ddt), mRNA	Enzyme	NM_024131
2	0	Rattus norvegicus kynurenine aminotransferase II (Kat2), mRNA	Enzyme	NM_017193
2	0	Rattus norvegicus mRNA for H(+)-transporting ATPase, complete cds	Enzyme	D10874
2	0	Rattus norvegicus NG,NG dimethylarginine dimethylaminohydrolase (Ddah1), mRNA	Enzyme	D86041
2	0	Rattus norvegicus ornithine aminotransferase (Oat), mRNA	Enzyme	NM_022521
2	0	Rattus norvegicus protein disulfide isomerase, mRNA	Enzyme	NM_012998
2	0	Rattus norvegicus insulin-like growth factor binding protein 1 (Igfbp1), mRNA	Growth factor-related	NM_013144
2	0	Mus musculus destrin (Dsn-pending), mRNA	Cytoskeletal, membrane protein	NM_019771
2	0	Mus musculus presenilin-1 gene, alternatively spliced transcripts, complete cds	Cytoskeletal, membrane protein	AF007560
2	0	Rat brain calbindin-d28k (CaBP28K), mRNA	Cytoskeletal, membrane protein	M27839
2	0	Rattus norvegicus mRNA for neuronal beta-catenin like protein (ORF1)	Cytoskeletal, membrane protein	AJ301634
2	0	Rattus sp. liver tricarboxylate carrier, mRNA	Mitochondria	S70011

Table 2. Continued.

Frequency NR	Sham-operated	Gene name	Category	Accession number
2	0	Mus musculus adult male kidney cDNA, RIKEN fclone:0610007F03	Mitochondria	AK018712
2	0	Mus musculus histone deacetylase 5 (Hdac5), mRNA	Nuclear	NM_010412
2	0	Rattus norvegicus mRNA for ribonucleoprotein F, complete cds	Nuclear	NM_022397
2	0	Human DNA sequence from clone 486I3 on chromosome 6q22.1-22.3	Nuclear	AL050331
2	0	Homo sapiens 5-HT receptor, mRNA, complete cds	Receptor channel	AF251055
2	0	Mus musculus chloride intracellular channel 4 (mitochondrial) (Clic4), mRNA	Receptor channel	NM_013885
2	0	Homo sapiens cDNA FLJ13989 fis, clone Y79AA1002083	Ribosome	AK024051
2	0	Homo sapiens, clone IMAGE:2820942, mRNA, partial cds	Ribosome	BC006474
2	0	Mus musculus N-myc downstream regulated 3 (Ndr3), mRNA	Signal transduction	NM_013865
2	0	Mus musculus pyruvate kinase 2 (pkm2), mRNA	Signal transduction	NM_011099
2	0	Rat casein kinase I delta, mRNA, complete cds	Signal transduction	L07578
2	0	Rattus norvegicus 14-3-3-zeta isoform (Prkcz), mRNA	Signal transduction	U37252
2	0	Rattus norvegicus mRNA for inositol hexakisphosphate kinase, complete cds	Signal transduction	AB049151
2	0	Rattus norvegicus rap7a (Rap7a), mRNA	Signal transduction	NM_022526
2	0	R.norvegicus mRNA for monocarboxylate transporter	Transporter	X86216
2	0	Mus musculus 10, 11 days embryo cDNA, RIKEN clone:2810413P16	Unknown	BC011420
2	0	Mus musculus 10 days embryo cDNA, RIKEN clone:2610315D18	Unknown	AK012030
2	0	Rattus norvegicus 4 BAC CH230-5L13	Unknown	AC126722
2	0	Rattus norvegicus similar to hypothetical protein MGC14421	Unknown	AC126722
2	0	Mus musculus ES cells cDNA, RIKEN clone:2410083E08	Unknown	AK010737
2	0	Novel gene 1	Novel	
2	0	Novel gene 2	Novel	
2	0	Novel gene 3	Novel	

The mRNAs, which were more frequent in the 5/6 nephrectomized kidney database compared with sham-operated kidney database, were listed. Significant difference was defined when the frequency of 5/6 nephrectomized kidney database was threefold higher than that of sham-operated kidney database and the subtractive difference was more than two.

The residual kidneys, which were removed necrotic region, were actually enlarged twofold in the 5/6 nephrectomized rats, as the weights of the 5/6 nephrectomized kidney were about one third those of the sham-operated kidneys despite 5/6 ablations.

The results of Northern blotting and Western blotting are shown in Figure 1. The mRNA expression of cathepsin B, osteopontin, kidney injury molecule-1 (KIM-1), and insulin-like growth factor binding protein-1 (IGFBP1) was significantly increased in the 5/6 nephrectomized kidney (Fig. 1A). The mRNA expression of OAT1, Na⁺-K⁺ATPase α 1 subunit, elongation factor 1 α , glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and aquaporin 2 was maintained in the 5/6 nephrectomized kidney (Fig. 1B). However, the mRNA expression of phenylalanine hydroxylase, 4F2hc, and OCTN2 was significantly decreased in the 5/6 nephrectomized kidney (Fig. 1C). These results were well comparable with the in silico subtractive database (Tables 2 and 3). In addition, the protein expression of cathepsin B, osteopontin, OAT1, and Na⁺-K⁺ATPase α 1 subunit were also comparable with the in silico subtractive database (Fig. 1).

Novel or unknown genes differentially expressed between the 5/6 nephrectomized and sham-operated kidneys

There were eight novel or unknown genes appearing twice in the 5/6 nephrectomized and 0 times in the sham-operated kidney database (Table 2). Among them, the expression of two genes designated UR-NR (up-regulated in the 5/6 nephrectomized kidney) #1 (GenBank Accession No. AB108667) and #2 (GenBank Accession No. AB108668) (the nucleotide sequences reported in this article have been submitted to GenBank with accession numbers between AB108667 and AB108671) was significantly increased in the 5/6 nephrectomized kidney as determined by Northern blotting (Fig. 2A and B). There were 10 novel or unknown genes appearing twice in the sham-operated and 0 times in the 5/6 nephrectomized kidney database (Table 3). Among them, three genes designated DR-NR (down-regulated in the 5/6 nephrectomized kidney) #1 (GenBank Accession No. AB108669), #2 (AB108670), and #3 (AB108671) had a significantly decreased expression in the 5/6 nephrectomized kidney as shown by Northern blotting (Fig. 2C to E).

Table 3. The mRNAs down-regulated in the 5/6 nephrectomized (NR) kidney database by in silico subtraction

Frequency NR	Sham-operated	Gene name	Category	Accession number
0	7	Rattus norvegicus phenylalanine hydroxylase (Pah), mRNA	Enzyme	NM_012619
2	7	Rattus norvegicus uromodulin (Tamm-Horsfall protein) (Umod), mRNA	Other	NM_011044
1	7	Mus musculus phosphoenolpyruvate carboxykinase 1, cytosolic (Pck1), mRNA	Enzyme	NM_017082
2	6	Rattus norvegicus cytochrome P450 arachidonic acid epoxidase, mRNA	Metabolic enzyme	U04733
0	5	Rattus norvegicus intestinal type II membrane glycoprotein 4F2hc, mRNA	Transporter	U59324
1	5	Mus musculus, similar to glutathione S-transferase theta 1, clone MGC:6769, mRNA	Enzyme	BC003903
0	4	Rattus norvegicus acyl-coenzyme A dehydrogenase, C-4 to C-12 straight-chain, mRNA	Enzyme	NM_013113
0	4	Rattus norvegicus pyruvate dehydrogenase kinase 2 subunit p45 (PDK2) (Pdk2), mRNA	Signal transduction	NM_016986
0	4	Rattus norvegicus ATPase Na ⁺ /K ⁺ transporting beta 1 polypeptide (Atp1b1), mRNA	Transporter	NM_030872
1	4	Rat mRNA for cerebellar Ca-binding protein, spot 35 protein	Cytoskeletal, membrane protein	NM_031984
0	3	Rattus norvegicus calreticulin (Calr), mRNA	Other	NM_022399
0	3	Rattus norvegicus spp-24 precursor, mRNA, partial cds	Other	NM012570
0	3	Rattus norvegicus glutamate dehydrogenase (Glud1), mRNA	Enzyme	U19485
0	3	Rattus norvegicus thyroxine deiodinase, type I (Dio1), mRNA	Enzyme	NM_012653
1	3	Mus musculus, similar to NADH dehydrogenase Fe-S protein 2, clone IMAGE:3600832	Enzyme	BC003898
1	3	Rat mRNA for gamma-glutamyl transpeptidase (EC 2.3.2.2)	Enzyme	X15443
1	3	Rattus norvegicus methylmalonate semialdehyde dehydrogenase gene (Mmsdh), mRNA	Enzyme	AF359355
1	3	Rattus norvegicus retinol dehydrogenase type II, mRNA, complete cds	Enzyme	NM_031057
1	3	Rattus norvegicus superoxide dismutase 3 (Sod3), mRNA	Enzyme	U33500
1	3	Rattus norvegicus membrane-bound aminopeptidase P, mRNA, complete cds	Protease	NM_012880
0	2	Rattus norvegicus (rsec6), mRNA, complete cds	Other	U32575
0	2	Rattus norvegicus transcobalamin II precursor (Tcn2p), mRNA	Other	NM_022534
0	2	Rattus norvegicus prosaposin (sulfated glycoprotein, sphingolipid hydrolase activator)	Other	NM_013013
0	2	Homo sapiens, calcium binding atopy-related autoantigen 1, clone MGC:4521, mRNA	Other	BC004216
0	2	Mus musculus interferon alpha responsive gene, 15 kD (Ifrg15-pending), mRNA	Other	NM_022329
0	2	Mus musculus Mpv17-like protein, mRNA, complete cds	Other	AF305634
0	2	Rattus norvegicus src family associated phosphoprotein 2 (Scap2), mRNA	Other	NM_130413
0	2	Mus musculus, similar to autocrine motility factor receptor, clone IMAGE:3500628, mRNA	Receptor channel	BC003256
0	2	Rattus norvegicus hydroxysteroid dehydrogenase, 11 beta type 1 (Hsd11b1), mRNA	Enzyme	NM_017080
0	2	Rattus norvegicus pyruvate carboxylase, mRNA, complete cds	Enzyme	U36585
0	2	Hprt = hypoxanthine phosphoribosyltransferase [rats, mRNA, 853 nt]	Enzyme	S79292
0	2	Rattus sp. mRNA for argininosuccinate lyase, complete cds	Enzyme	D13978
0	2	Rattus norvegicus tissue-type transglutaminase (TgaseII), mRNA, complete cds	Enzyme	AF106325
0	2	Rattus norvegicus cysteine-sulfinatase decarboxylase (Csad), mRNA	Enzyme	NM_021750
0	2	Rattus norvegicus Glutamate oxaloacetate transaminase 2, mitochondrial (Got2), mRNA	Enzyme	NM_013177
0	2	Homo sapiens heat shock protein 75 (TRAP1), mRNA	HSP	NM_016292
0	2	Rattus norvegicus vesicle-associated membrane protein 7 (Vamp7) mRNA, complete cds	Cytoskeletal, membrane protein	AF281632
0	2	Homo sapiens, Similar to membrane protein of cholinergic synaptic vesicles, mRNA	Cytoskeletal, membrane protein	XM_213485
0	2	Rat mRNA for mitochondrial long-chain 3-ketoacyl-CoA thiolase beta-subunit	Mitochondria	D16479
0	2	Mus musculus SMT3 (suppressor of mif two, 3) homolog 1 (S. cerevisiae) (Smt3h1), mRNA	Nuclear	NM_019929
0	2	Mus musculus NUDE-like protein mRNA, complete cds	Nuclear	AF323918
0	2	Mus musculus microtubule-associated protein 7 (Mtap7), mRNA	Nuclear	NM_008635
0	2	Mus musculus proteasome (prosome, macropain) 28 subunit, 3 (Psmc3), mRNA	Protease	NM_011192
0	2	Rattus mRNA for ribosomal protein S2	Ribosome	X57432
0	2	Homo sapiens sorting nexin 17 (SNX17), mRNA	Signal transduction	NM_014748
0	2	Rattus norvegicus solute carrier family 25, member 1 (Slc25a1), mRNA	Transporter	NM_172577
0	2	Rattus norvegicus solute carrier family 12, member 1 (Slc12a1), mRNA	Transporter	NM_019134
0	2	Rattus norvegicus organic cation/carnitine transporter (OCTN2) mRNA	Transporter	AF110416
0	2	Rattus norvegicus Glutathione-S-transferase, alpha type 2 (Gsta2), mRNA	Enzyme	NM_017013
0	2	Rattus norvegicus similar to hypothetical protein FLJ20356, mRNA	Unknown	XM_223527
0	2	Mus musculus RIKEN cDNA 221040O07 gene, mRNA	Unknown	BC036156
0	2	Mus musculus adult male liver cDNA, RIKEN clone:1300002B20, full insert sequence	Unknown	AK004845
0	2	Mus musculus ES cells cDNA, RIKEN clone:2410074K14, full insert sequence	Unknown	AK010721
0	2	Mouse DNA sequence from clone RP23-22K4 on chromosome 2, complete sequence	Unknown	AL663077
0	2	Homo sapiens hypothetical protein DKFZp564B1162 (DKFZP564B1162), mRNA	Unknown	NM_031305
2	0	Mus musculus 13 days embryo head cDNA, RIKEN clone:3110030P16	Unknown	XM_055115
0	2	Mus musculus NN5H6H tumor-related protein, mRNA, complete cds	Unknown	AY035213
0	2	Novel gene 4	Novel	
0	2	Novel gene 5	Novel	

The mRNAs, which were less frequent in the 5/6 nephrectomized kidney database compared with sham-operated kidney database, are listed. Significant difference was defined when the frequency of 5/6 nephrectomized kidney database was threefold lower than that of sham-operated kidney database and the subtractive difference was more than two.

Table 4. The list of genes subjected to confirmation of the database

	Gene name	Frequency		Category	Accession number
		NR	Sham		
NR > sham	Cathepsin B	4	0	Protease	NM022597
	Osteopontin	3	0	Cytoskeletal, membrane protein	NM012881
	KIM-1	3	0	Cytoskeletal, membrane protein	AF035963
	IGF binding protein1	2	0	Growth factor-related	NM013144
NR = sham	OAT1	2	4	Transporter	AF008221
	Na ⁺ -K ⁺ ATPase α 1	2	2	Transporter	NM012504
	Elongation factor -1 α	12	14	Ribosome	X61043
	GAPDH	4	6	Enzyme	NM017008
	Aquaporin 2	2	2	Transporter	D13906
NR < sham	Pah	0	7	Enzyme	NM 012619
	4F2hc	0	5	Transporter	U59324
	OCTN2	0	2	Transporter	AF110416

Abbreviations are: Accession number, number in Genbank database; frequency, times appeared in 1033 or 1015 sequences; KIM-1, kidney injury molecule-1; IGF, insulin like growth factor; OAT-1, organic anion transporter 1; Pah, phenylalanine hydroxylase; OCTN2, novel organic cation transporter 2. The expressions of four genes which were more frequent in the 5/6 nephrectomized (NR) than sham-operated (sham) kidney, five genes which were equally frequent in the 5/6 nephrectomized and sham-operated kidney, and three genes which were less frequent in the 5/6 nephrectomized than sham-operated kidney, were examined by Northern blotting or Western blotting. Nucleotide positions of probes are from the sequences published in GenBank/EBI Data Bank.

To obtain more information about these five genes, the expression levels in the earlier phases of 5/6 nephrectomized kidney, the tissue distribution, the localization along the microdissected nephron segments, and the expression levels in the drug-induced acute nephropathy models were examined. Blood and urine biochemical parameters and kidney weights are shown in Table 5. Creatinine clearance of 5/6 nephrectomized rats decreased to 2.1 mL/min/kg at 3 days after the operation, and this level was maintained until 2 weeks. On the other hand, compensatory hypertrophy was not observed in 5/6 nephrectomized kidneys 3 days after the operation, and kidney weight was increased sequentially according to the compensatory hypertrophy thereafter. Figure 2 shows the mRNA expression levels of the five unknown genes in sham-operated and 5/6 nephrectomized kidneys 3 days, 1 week, and 2 weeks after subtotal nephrectomy. The mRNA expression of UR-NR #1 of 5/6 nephrectomized kidney was more markedly increased at 1 week than at 2 weeks after the operation (Fig. 2A). The mRNA expression of UR-NR #2 of 5/6 nephrectomized kidney was maintained at 3 days after the operation but increased sequentially thereafter (Fig. 2B). The mRNA expression of DR-NR #1 and DR-NR #3 of 5/6 nephrectomized kidney was maintained at 3 days and 1 week after the operation and decreased at 2 weeks after the operation (Fig. 2C and E). The mRNA expression of DR-NR #2 of 5/6 nephrectomized kidney was equally decreased at 1 week and 2 weeks after the operation (Fig. 2D).

Figure 3 shows the mRNA distribution of the five unknown genes in eight tissues. UR-NR #1 mRNA was detected in all tissues examined except for the small intestine and spleen. UR-NR #2 and DR-NR #1 mRNAs were strongly detected in the spleen and kidneys, but weakly detected in other tissues. DR-NR #2 mRNA existed predominantly in the kidneys. DR-NR #3 mRNA was detected only in the kidney and liver.

mRNA localization of the five unknown genes along the nephron segments in sham-operated (Fig. 4A) and 5/6 nephrectomized kidneys (Fig. 4B) was examined. UR-NR #1 mRNA was localized at IMCD in the sham-operated kidneys. It was detected not only in IMCD but also in glomerulus, PCT, and PST in 5/6 nephrectomized kidneys. UR-NR #2 mRNA was not detected either in sham-operated or 5/6 nephrectomized renal nephron segments, and was thought to exist in the interstitium or blood vessels in the kidneys. DR-NR #1 mRNA was detected ubiquitously both in sham-operated and 5/6 nephrectomized renal nephron segments except in MAL. DR-NR #2 mRNA was localized at glomerulus, PCT, and the collecting duct in sham-operated kidneys, but was only localized at PCT and IMCD in 5/6 nephrectomized kidneys. DR-NR #3 mRNA was detected predominantly in the proximal tubules in sham-operated kidneys, and its localization shifted to glomerulus or MAL in 5/6 nephrectomized kidneys.

The mRNA expression levels of the five unknown genes were also examined in acute renal insufficiency induced by the administration of cisplatin or vancomycin. Blood and urine biochemical parameters and kidney weights are shown in Table 6. In the cisplatin nephropathy, a marked reduction of renal function, decrease in creatinine clearance, increases in BUN and urinary NAG excretion, and albuminuria were observed. However, there was no change in kidney weight. In contrast to the cisplatin nephropathy, there was no change in creatinine clearance and only a slight increase of BUN in the vancomycin nephropathy. Although urinary NAG excretion was increased, albuminuria did not appear in the vancomycin nephropathy. The kidney weight of the animals with vancomycin nephropathy was markedly increased. The results of Northern blotting are shown in Figure 5. The mRNA expression of UR-NR #1 was significantly increased in the vancomycin but not the cisplatin

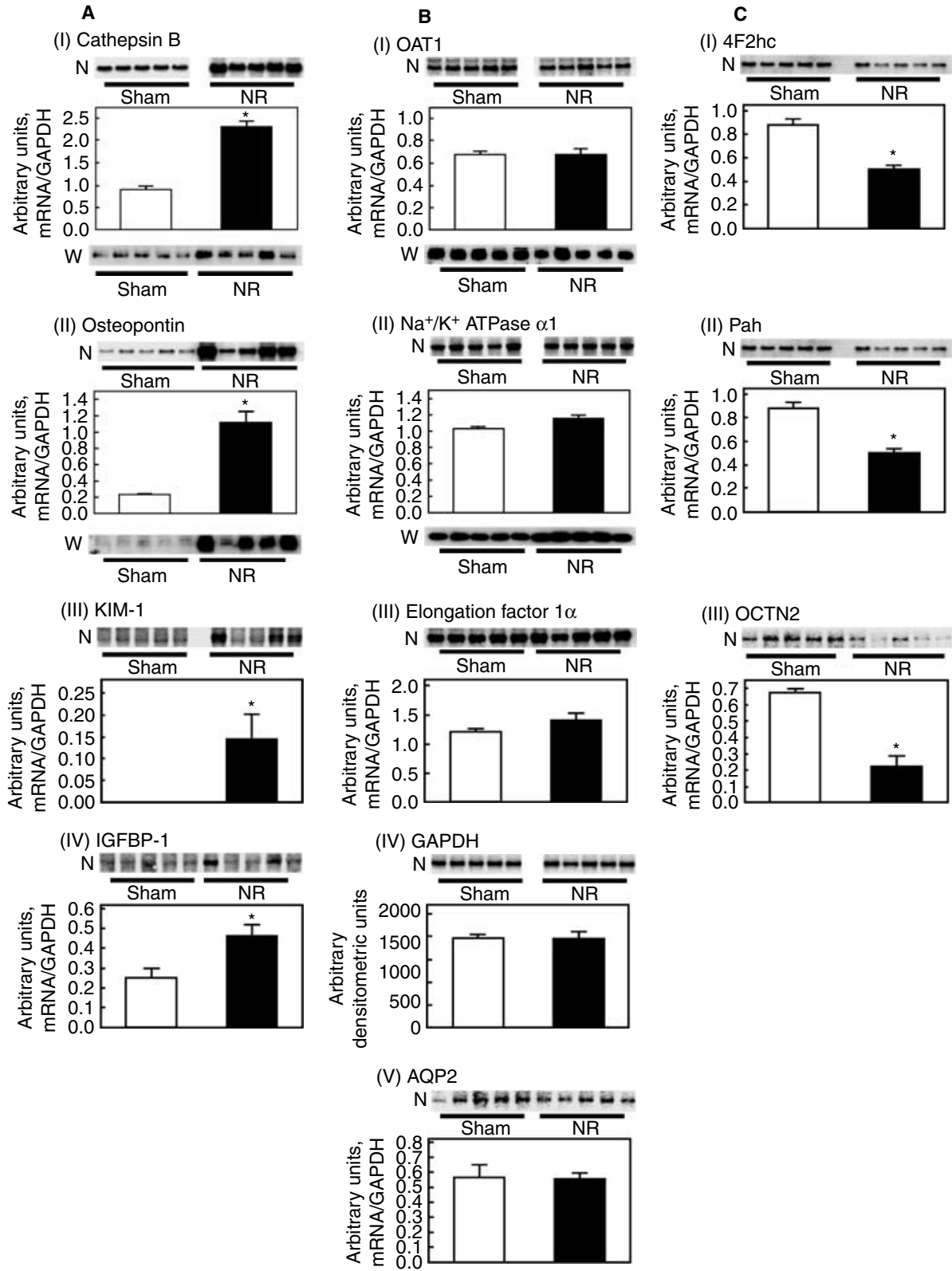


Fig. 1. Northern blotting or Western blotting of the genes up-regulated (A), maintained (B), or down-regulated (C) in the 5/6 nephrectomized (NR) kidney in the subtractive database. Five micrograms of total RNA of kidneys was hybridized with each probe under high stringency conditions. The protein expressions of cathepsin B, osteopontin, OAT1, and Na⁺-K⁺ ATPase α 1 subunit, whose antibodies were available, were also examined by Western blotting. The amounts of mRNA were quantified by densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each column represents the mean \pm SEM of five rats. * P < 0.05, significantly different from sham (Student unpaired t test). Abbreviations are: N, Northern blotting; W, Western blotting; KIM-1, kidney injury molecule-1; IGFBP-1, insulin like growth factor binding protein-1; OAT1, organic anion transporter1; AQP2, aquaporin 2; Pah, phenylalanine hydroxylase; OCTN2, novel organic cation transporter 2.

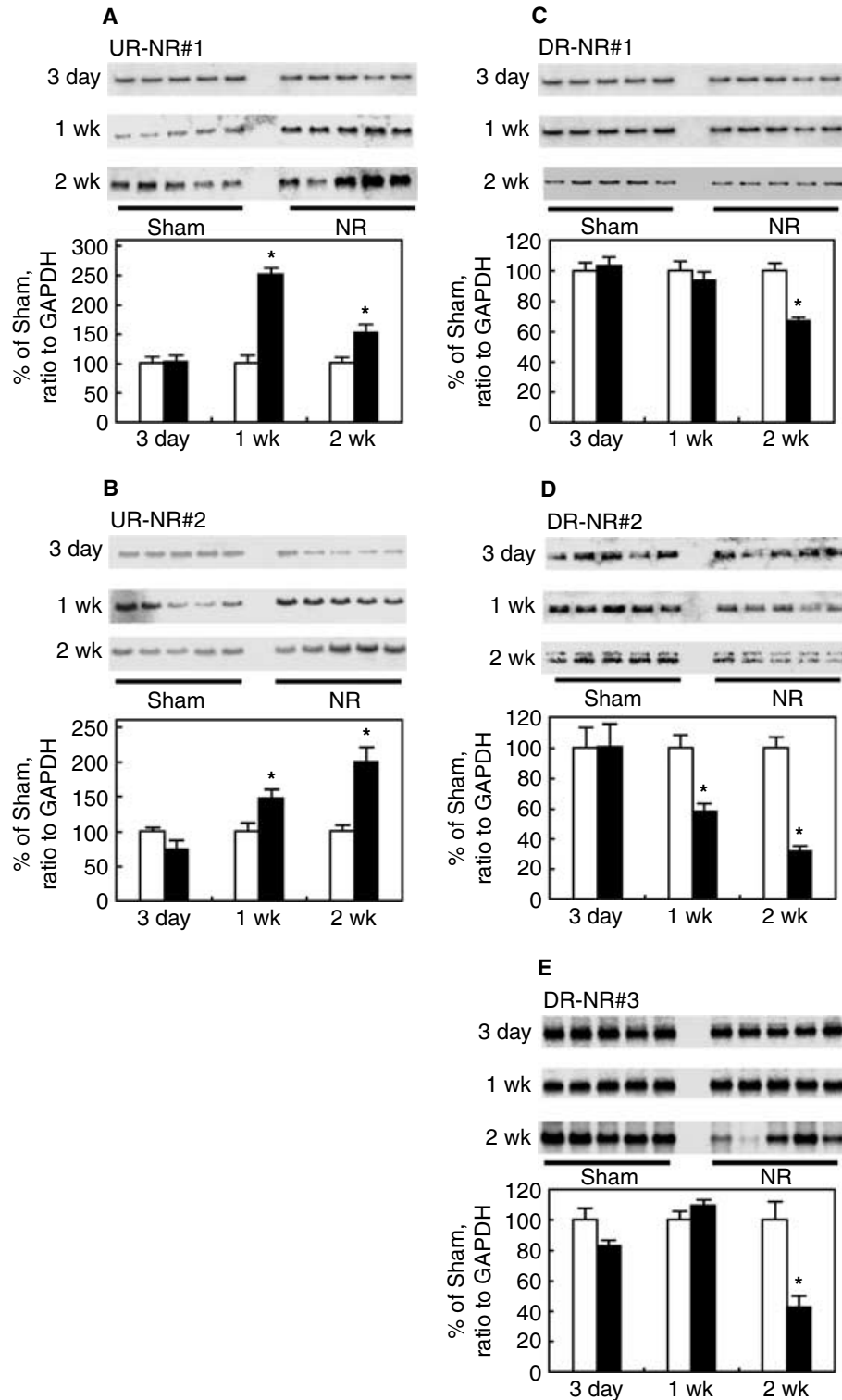


Fig. 2. Northern blotting of the five unknown genes in the 5/6 nephrectomized (NR) and sham-operated (sham) kidneys. mRNA expression of up-regulated 5/6 nephrectomized (UR-NR) #1 (A), UR-NR #2 (B), down-regulated 5/6 nephrectomized (DR-NR) #1 (C), DR-NR #2 (D), and DR-NR #3 (E) in the 5/6 nephrectomized and sham-operated kidneys 3 days, 1 week, and 2 weeks after the operation was examined. Five micrograms of total RNA of kidneys was hybridized with each probe under high stringency conditions. The amounts of mRNA were quantified by densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each column represents the mean \pm SEM of five rats. * $P < 0.05$, significantly different from sham-operated (Student unpaired t test).

Table 5. State of renal function of the 5/6 nephrectomized (NR) and sham-operated (sham) rats 3 days, 1 week, and 2 weeks after subtotal nephrectomy

		Body weight g	Plasma creatinine mg/dL	Blood urea nitrogen mg/dL	Creatinine clearance mL/min/kg	Urinary excretion of albumin mg/day	Urinary excretion of NAG U/day	Kidney weight g
Sham	3 days	204 ± 2	0.4 ± 0.0	14 ± 0	7.0 ± 0.5	0.2 ± 0.0	156 ± 15	1.6 ± 0.0
	1 week	232 ± 3	0.5 ± 0.0	11 ± 0	5.1 ± 0.4	0.4 ± 0.1	232 ± 19	1.7 ± 0.0
	2 weeks	270 ± 4	0.5 ± 0.0	14 ± 2	5.2 ± 0.1	0.3 ± 0.1	211 ± 16	1.7 ± 0.0
NR	3 days	188 ± 3 ^a	1.4 ± 0.1 ^a	56 ± 2 ^a	2.1 ± 0.3 ^a	6.9 ± 1.7 ^a	180 ± 44	0.3 ± 0.0 ^a
	1 week	192 ± 5 ^a	1.1 ± 0.1 ^a	48 ± 2 ^a	2.1 ± 0.1 ^a	7.1 ± 2.2 ^a	177 ± 15	0.4 ± 0.0 ^a
	2 weeks	230 ± 10 ^a	1.2 ± 0.1 ^a	59 ± 6 ^a	1.9 ± 0.1 ^a	4.0 ± 2.2 ^a	187 ± 11	0.6 ± 0.0 ^a

NAG is *N*-acetyl-β-D-glucosaminidase.

The urine and blood samples were collected at 3 days, 1 week, and 2 weeks after the operation. Data represent the mean ± SEM of five rats.

^a*P* < 0.05, significantly different from sham (Student unpaired *t* test).

nephropathy. The mRNA expression of DR-NR #2 and #3 was significantly decreased in the cisplatin, but not the vancomycin nephropathy. There were no changes in the mRNA expression of UR-NR #2 and DR-NR #1 among the three treated groups.

Structure of unknown genes

UR-NR #1, UR-NR #2, DR-NR #1, DR-NR #2, and DR-NR #3 were fully isolated by the 5' RACE technique. They were all highly homologous to the mouse or human putative clone in which only nucleic acid sequences were identified. Their deduced amino acid sequences were also highly homologous to the putative proteins corresponding to the putative nucleotide (Table 7). Figure 6 shows their deduced amino acid sequences, and the conserved domains are listed in Table 8. The topologies of these five proteins were predicted by SOSUI system, which is a tool for secondary structure prediction of membrane proteins (www.sosui.proteome.bio.taut.ac.jp, Mitaku and Hirokawa, 1999). DR-NR #3 was found to be a membrane-bound protein. DR-NR #1 was predicted to be a secreted protein, but the other three proteins were thought to be soluble proteins. UR-NR #1 consisted of 1075 bp nucleotides, and contained an open reading frame of 117 amino acids. UR-NR #1 did not show any significant similarity with known genes and conserved domains (Fig. 6A). UR-NR #2 consisted of 3752 bp nucleotides, and contained an open reading frame of 353 amino acids. A conserved domain search revealed that UR-NR #2 had a Tre/Bub2/Cdc16 (TBC) domain, which was the guanosine triphosphatase (GTPase) activator protein of Rab-like small GTPases (Rab-GAPs), regulators of intracellular vesicle trafficking (Fig. 6B) [27]. DR-NR #1 consisted of 3315 bp nucleotides, contained an open reading frame of 297 amino acids, and had a conserved domain of isochorismatase, a family of hydrolase enzymes (Fig. 6C) [28]. DR-NR #2 consisted of 3032 bp nucleotides, and contained an open reading frame of 596 amino acids (Fig. 6D). DR-NR #2 had a conserved domain of Rho-GAPs, which negatively regulated Rho [29,

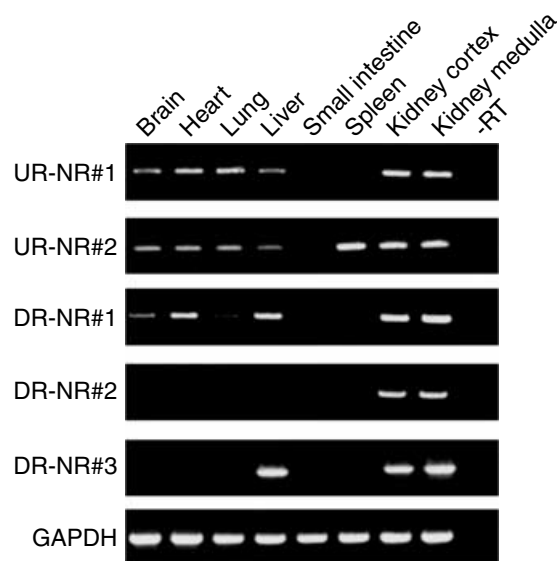


Fig. 3. Detection of mRNA of the five unknown genes in rat tissues by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA (5 µg) from the tissues indicated was reverse-transcribed, and the cDNA synthesized was amplified using a set of primers specific for each clone. The PCR products were separated by electrophoresis through 1% agarose gels and stained with ethidium bromide. Abbreviations are: –RT, whole kidney without reverse transcriptase; UR-NR #1, up-regulated 5/6 nephrectomized #1; UR-NR #2, up-regulated 5/6 nephrectomized #2; DR-NR #1, down-regulated 5/6 nephrectomized #1; DR-NR #2, down-regulated 5/6 nephrectomized #2; DR-NR #3, down-regulated 5/6 nephrectomized #3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

30]. DR-NR #3 consisted of 1753 bp nucleotides, and contained an open reading frame of 324 amino acids (Fig. 6E). DR-NR #3 contained a typical NADP⁺-binding motif (TGxxxGxG) and a short chain dehydrogenase active site motif (S-Y-K) [31]. In addition, 101 amino acids of DR-NR #3, from 89 to 189, were very similar to “hypertension-induced rat S2 protein,” which was previously reported to be up-regulated in spontaneously hypertensive rats [32]. The similarity was approximately 98% in the matched region, totaling 31% homology (Fig. 6E). Therefore, DR-NR #3 would be a full length of rat S2 protein.

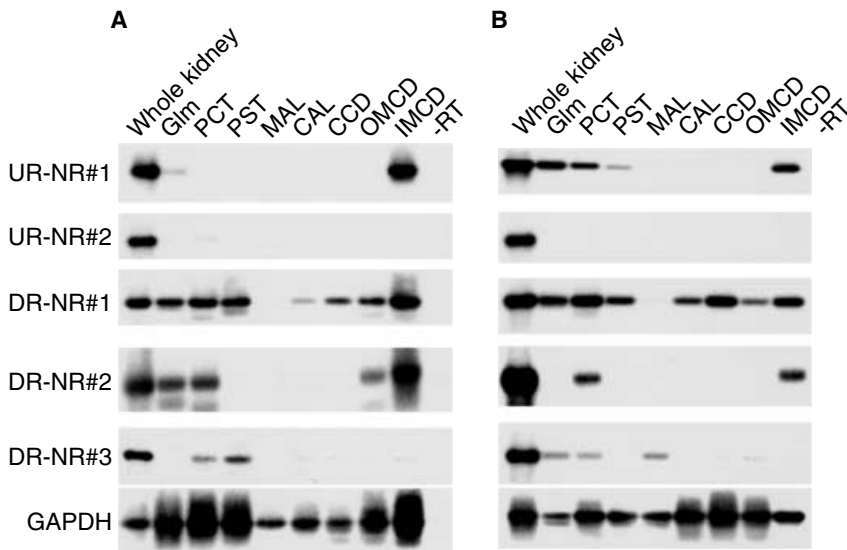


Fig. 4. Detection of mRNA of the five unknown genes in nephron segments in sham (A) and 5/6 nephrectomized (NR) kidney (B) 2 weeks after the operation by reverse transcription-polymerase chain reaction (RT-PCR) and subsequent Southern blotting. Each PCR amplification (35 cycles) was performed using a part of the reverse transcription reaction derived from 20 glomeruli or 8 mm length of renal tubule. The cDNA synthesized was amplified using sets of primers specific for each clone. The PCR products were separated by electrophoresis through 1.0% agarose gels. The agarose gels were transferred onto a nylon membrane and hybridized with the [32 P] deoxycytidine triphosphate (dCTP)-labeled each cDNA as probes at high stringency. Abbreviations are: Glm, glomerulus; PCT, proximal convoluted tubule; PST, proximal straight tubule; MAL, medullary thick ascending limb; CAL, cortical thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; -RT, whole kidney without reverse transcriptase; UR-NR #1, up-regulated 5/6 nephrectomized #1; UR-NR #2, up-regulated 5/6 nephrectomized #2; DR-NR #1, down-regulated 5/6 nephrectomized #1; DR-NR #2, down-regulated 5/6 nephrectomized #2; DR-NR #3, down-regulated 5/6 nephrectomized #3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 6. State of renal function of rats treated with cisplatin or vancomycin

	Body weight g	Plasma creatinine mg/dL	Blood urea nitrogen mg/dL	Creatinine clearance mL/min/kg	Urinary excretion of albumin mg/day	Urinary excretion of NAG U/day	Kidney weight g
Vehicle	333 \pm 8	0.7 \pm 0.0	13 \pm 2	3.2 \pm 0.1	0.8 \pm 0.3	277 \pm 32	2.2 \pm 0.1
Cisplatin	268 \pm 5 ^a	2.7 \pm 0.3 ^a	105 \pm 15 ^a	0.5 \pm 0.1 ^a	3.5 \pm 0.8 ^a	1093 \pm 241 ^a	2.2 \pm 0.1
Vancomycin	320 \pm 11	0.8 \pm 0.1	28 \pm 4 ^a	3.3 \pm 0.4	1.9 \pm 0.8	582 \pm 41 ^a	2.9 \pm 0.2 ^a

NAG is *N*-acetyl- β -D-glucosaminidase.

Rats were treated with cisplatin (6 mg/kg) intraperitoneally, vancomycin (500 mg/kg) intravenously or vehicle (saline) intravenously. Urine and blood samples were collected 3 days after the treatments. Data represent the mean \pm SEM of five rats.

^a*P* < 0.05, significantly different from vehicle (Fisher's *t* test).

DISCUSSION

Large-scale cDNA expression profiling was performed to construct the expression profiles of 5/6 nephrectomized and sham-operated kidney. In the 5/6 nephrectomized kidney, the expression of growth factor-related mRNA such as for IGF, IGFBPs, and TGF- β , which are known to directly up-regulate cell growth was increased compared to that in the sham-operated kidney (Table 1) [3, 6, 33, 34]. It is known that compensatory hypertrophy occurs at 2 weeks after nephrectomy [2]. The increase in the expression of growth factor-related genes might correspond to the compensatory hypertrophy. The mRNA-encoded cytoskeletal or membrane proteins were also increased in the 5/6 nephrectomized kidney compared to the sham-operated kidney. The increases of these mRNAs were thought to be due to the process of tissue repair and hypertrophy. In contrast to the above genes, the mRNA expression frequency related to transporters and cofactors of transporters such as OCTN2 and 4F2hc, was decreased

in the 5/6 nephrectomized kidney (Table 1). Our previous reports demonstrated that the mRNA of the organic anion and the cation transporters, OAT-K and OCT2, was down-regulated, accompanied by a decrease in the urinary excretion of methotrexate and cimetidine, respectively, in the 5/6 nephrectomized rats [9, 10]. The decrease in transporter-related genes shown in this study might account for the reduction of urinary excretion of various drugs in CRF. Taken together, these changes observed in the 5/6 nephrectomized and in the sham-operated kidney databases could account for the physiologic state of the residual kidneys in 5/6 nephrectomized rats.

To confirm whether the *in silico* subtractive database of 5/6 nephrectomized kidney reflected the actual expression of mRNA or protein, the expression of 12 genes, which have been considered to play significant roles in the kidney, was examined by Northern blotting or Western blotting in the 5/6 nephrectomized and in the sham-operated kidney (Table 4). The differential expression

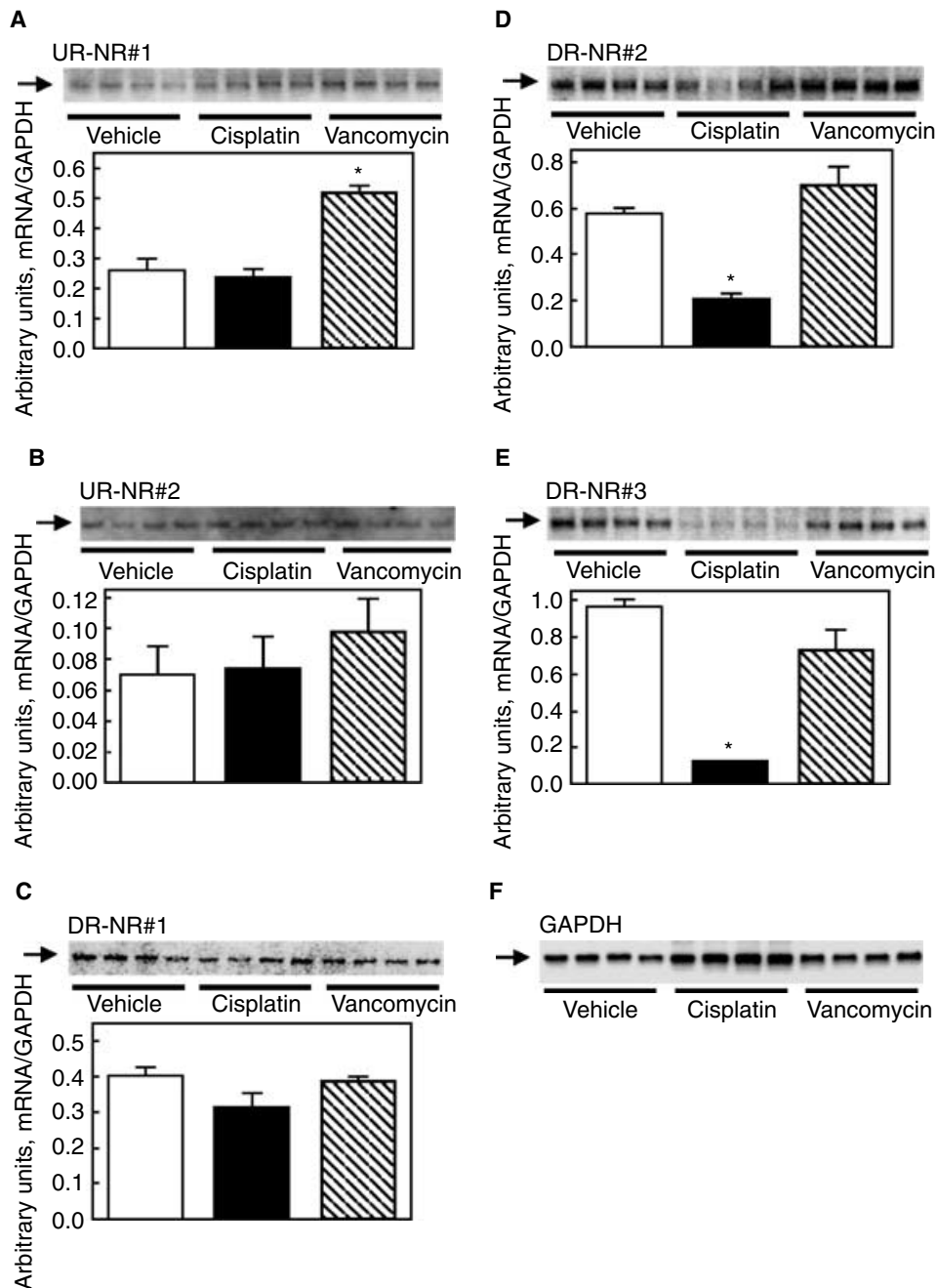


Fig. 5. Northern blotting of the five unknown genes in the rats treated with cisplatin or vancomycin. Expression of up-regulated 5/6 nephrectomized (UR-NR) #1 mRNA (A), up-regulated 5/6 nephrectomized (UR-NR) #2 mRNA (B), down-regulated 5/6 nephrectomized (DR-NR) #1 mRNA (C), down-regulated 5/6 nephrectomized (DR-NR) #2 mRNA (D), down-regulated 5/6 nephrectomized (DR-NR) #3 (E), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (F) in vehicle, cisplatin, or vancomycin-treated rat kidneys was examined. Five micrograms of total RNA of kidneys was hybridized with each probe under high stringency conditions. The amounts of mRNA were quantified by densitometry and normalized to GAPDH. Each column represents the mean \pm SEM of four rats. * $P < 0.05$, significantly different from vehicle-treated rats (Fisher's t test).

patterns of these 12 genes between the 5/6 nephrectomized and sham-operated kidney databases were comparable with the results of Northern blotting and Western blotting (Fig. 1). Thus, the results indicated that the frequency shown in this subtractive database was reli-

able enough to estimate actual expression of mRNA and protein.

Digital expression profiling has been used to analyze gene expression on a large scale [15–18]. With this technique, cDNA was digested by the restriction enzyme

Table 7. The orthologues of the five unknown putative proteins

Clone ID	Accession number	Gene name of orthologues	Homology	Accession number of orthologues	
				Nucleotide	Protein
UR-NR #1	AB108667	Mus musculus 10 days embryo putative protein	97%	AK012030	BAB27984
UR-NR #2	AB108668	Mus musculus clone MGC:11983	97%	BC011420	AAH11420
DR-NR #1	AB108669	Mus musculus NN5H6H tumor-related protein mRNA	99%	AY035213	AAK64607
DR-NR #2	AB108670	Homo sapiens hypothetical protein DKFZp564B1162	82%	NM_031305	NP_112595
DR-NR #3	AB108671	Homo sapiens CGI-86 protein	61%	XM_055115	XP_055115

The ortholog of the five unknown deduced amino acid proteins differentially expressed in the 5/6 nephrectomized kidney are listed. Homology was estimated by GENETYX-MAC, version 10.1.

A UR-NR#1

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1  MQKSCDENEGTPQNTPKADEGHPSDEPPQAGENLQASGENVREETDGLRGEPAEPSPE
61 PKEDTPARHLNPEEVIRGVDELERLREEIRRVNKFVLMHWKQRHSRSPYPVCFRP

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B UR-NR#2

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1  MVVQAKKRELKEAQRKKQLEERCKVEESIGNAVLTWNNEILPNWETMWCSSKVRDLWQ
61  GIPPSVRGKVSILAMGNEVNITHELFDICPARAKERMRSLSTGGSEVENEDAGFSAADRE
121 ASLELIKLDISRTFPNLCIFQQGGPYHDLMSILGAYTCYRPDVGTVQGMFIAAVLIIN
181 LDTADAFIAPSNLLNKPQMAFFRVDEHGLMLTYFAAFVFFFEENLPKLPFAHFKNNLTAD
241 IYLIIDWIFTLYSKSLPLDLACRIWDVFCRDGEEFLFR TALGILKLFEDILTMDFIHSAQ
301 FLTRLPELDPADDVFAAISTVQMSRNKKWAQVLSALQKDSREMEEGSPSVRD

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C DR-NR#1

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1  MAAAEPSVAALAGGGVGAGAPSGGVFVLCFVSVPFARPASVPHGAGYDVLIQKFLSLYGDQ
61  VDMHRKFVVQLFAEHWGQYVDLPKGFVAVSERCKLRVLPLQIQLTTLGNLTFFPSTVFFCCD
121 MQERFRPAIKYFGDIIISVGQRLQQAIRLIGIPVITTEQYYPKGLGSTVQEIIDLTVGKLVLP
181 KTKFSMVLPEVEAALAEIPGVRSVVLPFGVETHVCIQQTALVLRGIEVHIVADATSSRS
241 MMDRMFALERLARTGIIVTTSEAVPLQLVADKDHFKFEIQNLIKASAPESGLLSKV

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D DR-NR#2

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1  MLVEQCVDIFIRQGLKEEGLFRLPGQANLVKELQDAFDCGEEKSPFDSNTDVETVASLLKL
61  YLRELPEPVVYAKYEDFLSCATLLSKEEAGVKELTKQVKSPLPVVYNLLKYICRFLDE
121 VQSYSGVNMKMSAQNLAIVFGPNILRPKVEDPLTIMEGTVVVQQMLSMVMSKHDRLPFKDT
181 EPQSKPQEGPNSNNNDGHHKVTIGQLQNKNNNTKESPVRRCSWDKPEPQRSSMDNGSP
241 TALSGSKTNSPRNSIHKL DVSRSPPLTVKKNPAFNKGRGIVTNGSFSSSNGEGVEKTQTT
301 PNGSLQARRSSSLESSGKWTGTHSVQNGTVRMGYLNTDTLGNLSNGRSMWLPNGYVTLR
361 DNKQKEPGESESHNRLSTYDNVHQFSLMNLDDKHRVDSATWSTSCSEISLPENSNSCR
421 SSTTTCPEQDFYGGNFEDFVLGPPQDDLSPDYENKSDRRSVGGRRSRATSSSDNSDT
481 FPGNTSSNHSALHSLVSSLKQEMTKQKIEYESRIKSLQNRNLTEEMNLNHLDELQERK
541 KFTMIEIKMRNAERAKEDAERNDMLQKEMEQQFFSTFGDLTVEPRRSEGRNTIWIQ

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E DR-NR#3

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DR-NR#3 1 : MMGFEFWMLA LCALLLLVLG LLSFLWLDSD LTLRAAWMG QCPEQALADK VVWITGASSG
DR-NR#3 61 : IGEEELAFQLS KLGVLVLSA RRGQELERVK RRCLENGNLK EKDILVPLD LADTSSHDIA
Rat S-2 1 : VK RRSLENGNLK EKDILVPLD LADTSSHDIA
DR-NR#3 121 : TKTVLQEFGR IDILVNNGGV AHASLVNTN MDIFKVLIEV NYLGTVSLTK CMLPHMMERN
Rat S-2 33 : TKTVLQEFGR IDILVNNGGV AHASLVNTN MDIFKVLIEV NYLGTVSLTK CMLPHMMERN
DR-NR#3 181 : QGKIVVMKSL VGIVPRPLCS GYAASKLALR GFFDVLRTLE FDYPGITLSM ICPGPVHSNI
Rat S-2 93 : QGKIVVMKS 101
DR-NR#3 241 : FQNAFTGDFT ETRLPKIPLF KMETSRCVQL ILVSLANDLE DIWIANQPVL LRAYVWQYVP
DR-NR#3 301 : FRDWILQGRY GKYISKVLGI TWYR

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Fig. 6. The deduced amino acid sequences of the five unknown genes. (A) Up-regulated 5/6 nephrectomized (UR-NR) #1 encoded a putative protein of 117 amino acids that are shown in the one-letter code. (B) Up-regulated 5/6 nephrectomized (UR-NR) #2 encoded a putative protein of 353 amino acids that are shown in the one-letter code. The region in bold is highly homologous with the TBC domain. (C) Down-regulated 5/6 nephrectomized (DR-NR) #1 encoded a putative protein of 298 amino acids that are shown in the one-letter code. The region in bold is highly homologous with the conserved domain of isocholismatase. (D) Down-regulated 5/6 nephrectomized (DR-NR) #2 encoded a putative protein of 596 amino acids that are shown in the one-letter code. The region in bold is highly homologous with the conserved domain of Rho-GAPs. (E) Down-regulated 5/6 nephrectomized (DR-NR) #3 encoded a putative protein of 324 amino acids that are shown in the one-letter code. The sequences of TGxxxGxG (boxed with a thick line and in bold) indicate the NADPH binding site. The sequences of S-Y-K (bold region) indicate the short chain dehydrogenase active site motif. An alignment with rat S-2 protein is also presented. Conserved residues between two proteins are boxed.

Table 8. The conserved domains of the five unknown deduced amino acid proteins

Clone ID	Region	Definition	E value	Accession number
UR-NR #1 ^a	—	—	—	—
UR-NR #2	58–294	TBC domain	6.0E-42	pfam00566
DR-NR #1	109–265	Isochorismatase family	1.3E-07	pfam00857
DR-NR #2	1–173	RhoGAP domain	2.0E-44	pfam00620
DR-NR #3	48–294	Short chain dehydrogenase	2.3E-46	pfam00106

Motifs of the five unknown putative proteins were searched against the Smart and Pfam libraries.

^aThere was no conserved domain in UR-NR #1.

*Mbo*I and sequenced from the 3' end. However, in our study, cDNA libraries were constructed without cleaving using *Mbo*I, and the cDNA inserts were sequenced from the 5' end. In many cases the sequenced region from the 5' end is different in the same genes, because full-length cDNAs are not always isolated. However, the identity of two genes in which sequenced regions are different can be easily examined by homology search, because the number of enrolled genes in Genbank, EMBL, or DDBJ has markedly increased recently. In this study, the genes not identified by BLAST search were further sequenced from the 3' end to determine their identity. So, the sequence from the 5' end used in this study should be suitable for digital expression profiling. In a previous digital expression profiling, about 70% of mouse renal proximal tubule genes and renal collecting duct genes were novel genes unidentified by homology search [17, 18]. In contrast, only 17% of 2048 sequenced genes were novel in the present study (Table 1). It was reported that the non-coding regions of the 3' end are sometimes variant among species or tissues [35, 36]. This might be one of the reasons why there were so many novel genes not identified by BLAST search in previous studies. Consequently, the sequence from the 5' end appeared to be more useful for digital expression profiling.

Although a previous report mentioned that cleaving cDNA by *Mbo*I could normalize the cloning efficiencies reflecting the size or base composition of cDNA [15], we constructed cDNA libraries without cleaving to sequence from the 5' end. Nevertheless, this subtractive database of 5/6 nephrectomized kidney well reflected the actual expression of mRNA or protein as mentioned above. In addition, GAPDH mRNA, which is abundantly and ubiquitously expressed and often used as an internal control, appeared both in the 5/6 nephrectomized and sham-operated kidney databases four and six times, respectively (Table 4). However, abundantly appearing GAPDH was not observed in other digital expression profiles [15, 17, 18]. Although the reason for the difference between this study and the other studies remains unknown, the results of the present study

are thought to more reasonably reflect the actual mRNA expression.

A subtractive analysis of 5/6 nephrectomized mouse kidney using the cDNA-RDA technique has been reported by Zhang et al [19]. Several genes up-regulated in the 5/6 nephrectomized mouse kidney were successfully isolated, but genes previously known to increase after 5/6 nephrectomy were not isolated in their study. In contrast, in our study, KIM-1 and osteopontin mRNA, which were reported to increase upon renal injury [37–39], also increased in the 5/6 nephrectomized kidney database (Table 2). This subtractive database reflected previous reports on the 5/6 nephrectomized rat, and might be useful to search for the molecular mechanisms of CRF.

We successfully isolated five unknown genes expressed differently between in the 5/6 nephrectomized and sham-operated kidney by in silico subtraction (Fig. 2). The ratio of false positive clones that failed to show their up- or down-regulated expression was 70% (13 clones out of 18), which was much lower than that of the previous reports (over 80%) in which differential display was used [19, 40, 41]. Although all five are highly homologous to known human or mouse genes, little information is available concerning the functions and tissue distributions of these hypothetical genes. Their expression patterns in the earlier phase of 5/6 nephrectomized kidney and other renal failure models might be useful to speculate their roles in such conditions as hypertrophy of kidneys, tissue repair, blood pressure, and albuminuria. The precise physiologic roles of these five genes could not be discussed only from these expression data and the structure. However, it could be speculated that DR-NR #3 might serve as a biomarker for renal tubular injury by CRF, such as KIM-1, which is a transmembrane protein of proximal tubule and is thought to serve as a urinary marker for acute renal tubular injury [42]. In addition, UR-NR #2 and DR-NR #1 might be involved in the pathology induced by the reduction of renal mass, because the mRNA expression level of these two genes was maintained in the kidneys impaired by cisplatin or vancomycin (Fig. 5B and C). Particularly, UR-NR #2 might be involved in the compensatory hypertrophy, as it was sequentially increased in 5/6 nephrectomized kidneys in parallel to the kidney weights. Further physiologic or pathophysiologic studies, including the individual variations of expression levels, are needed to elucidate the roles of these five unknown genes in the progressive nephropathy.

Digital databases based on random sequencing reflect the mRNA expression profiles for relatively highly expressed genes [17, 18, 20]. Although we performed in silico subtraction only between 5/6 nephrectomized and sham-operated kidney databases, this methodology enables us to create disease-specific gene subsets using various disease models, as well as body mapping [15, 16].

CONCLUSION

We constructed subtractive expression databases of 5/6 nephrectomized and sham-operated kidney, which reflected the actual alterations of mRNA expression caused by subtotal nephrectomy, and was suggested to be useful for analysis of the molecular mechanisms of CRF. In addition, we successfully isolated five unknown genes expressed differently in the 5/6 nephrectomized kidney from *in silico* subtraction, two of which were up-regulated and three down-regulated. Although these five genes might be involved in the progression and/or restoration of CRF, further studies are needed to elucidate their roles in the pathogenesis of CRF.

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